10. Garthwaite, J. *Trends Neurosci.* **14,** 60–67 (1991).
11. Moncada, S., Palmer, R. M. J. & Higgs, E. A. *Pharmac. Rev.* **43,** 109–142 (1991).
12. Bredt, D. S. & Snyder, S. H. *Neuron* **8,** 3–11 (1992).
13. Nathan, C. *FASEB J.* **6,** 3051–3064 (1992).
14. Bredt, D. S. & Snyder, S. H. *A. Rev. Biochem.* **63,** 175–195 (1994).
15. Xie, Q. *et al. Science* **256,** 225–227 (1992).
16. Geller, D. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **90,** 3491–3494 (1993).
17. Nunokawa, Y., Ishuda, N. & Tanaka, S. *Biochem. biophys. Res. Commun.* **191,** 89–94 (1993).
18. Wood, E. R. & Berger, H. *Biochem. biophys. Res. Commun.* **191,** 767–774 (1993).
19. Garg, U. C. & Hassid, A. *J. clin. Invest.* **83,** 1774–1777 (1989).
20. Lepoivre, M. *et al. Biochem. biophys. Res. Commun.* **179,** 442–448 (1991).
21. Kwon, N. S., Stuehr, D. J. & Nathan, C. F. *J. exp. Med.* **174,** 761–767 (1991).
22. Hogan, M., Cerami, A. & Bucala, R. *J. clin. Invest.* **90,** 1110–1115 (1992).
23. Buchkovich, K. J. & Ziff, E. B. *Molec. biol. Cell* **5,** 1225–1241 (1994).
24. Peunova, N. & Enikolopov, G. *Nature* **364,** 450–453 (1993).
25. Dawson, T. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **88,** 7797–7801 (1991).
26. Hope, B. T. *et al. Proc. natn. Acad. Sci. U.S.A.* **88,** 2811–2814 (1991).
27. Bredt, D.S. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **87,** 682–685 (1990).
28. Hirsch, D. B. *et al. Curr. Biol.* **3,** 749–754 (1993).

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# A calcium-channel homologue required for adaptation to dopamine and serotonin in *Caenorhabditis elegans*

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**PROCESSING and storage of information by the nervous system requires the ability to modulate the response of excitable cells to neurotransmitter. A simple process of this type, known as adaptation or desensitization, occurs when prolonged stimulation triggers processes that attenuate the response to neurotransmitter. Here we report that the *Caenorhabditis elegans* gene *unc-2* is required for adaptation to two neurotransmitters, dopamine and serotonin. A loss-of-function mutation in *unc-2* resulted in failure to adapt either to paralysis by dopamine or to stimulation of egg laying by serotonin. In addition, *unc-2* mutants displayed behaviours similar to those induced by serotonin treatment. We found that *unc-2* encodes a homologue of a voltage-sensitive calcium-channel α-1 subunit. Expression of *unc-2* occurs in two types of neurons implicated in the control of egg laying, a behaviour regulated by serotonin. Unc-2 appears to be required in modulatory neurons to downregulate the response of the egg-laying muscles to serotonin. We propose that adaptation to serotonin occurs through activation of an Unc-2-dependent calcium influx, which modulates the postsynaptic response to serotonin, perhaps by inhibiting the release of a potentiating neuropeptide.**

To identify molecules involved in adaptation in the *C. elegans* nervous system, we searched for mutants that failed to adapt to neuroactive substances. We focused primarily on two biogenic amines, dopamine and serotonin. Both compounds are found in *C. elegans* neurons[1,2], and both have striking effects on nematode behaviour. Exogenous serotonin has several behavioural effects: stimulation of egg laying, inhibition of locomotion, stimulation of feeding, and activation of a specific step in the male mating program[2–4]. We found that dopamine treatment had at least two effects on *C. elegans* behaviour: inhibition of movement, and inhibition of egg laying (Table 1). Thus dopamine and serotonin had opposing effects on egg laying and similar effects on movement, although serotonin, unlike dopamine, also caused animals to move in a twisting, 'kinking' manner. Sets of neurons expressing either serotonin or dopamine have been identified[1], including the serotonergic hermaphrodite-specific neurons (HSNs)[5], required for egg laying, and the CP neurons, which also contain serotonin and are involved in male mating[4].

To determine whether *C. elegans* could adapt to these neurotransmitters, we tested the response to dopamine and serotonin after prolonged exposure. Treatment with 3 mg ml$^{-1}$ (16 mM) dopamine initially inhibited egg laying and locomotion in wild-type animals. However, animals treated with dopamine in this manner for 4 hours or more recovered the ability to move and lay eggs normally. These pretreated animals became resistant to inhibition of both egg laying and locomotion by up to 6 mg ml$^{-1}$ dopamine, indicating that they had adapted to dopamine (Table 1; Fig. 1*a*, *c*). Moreover, when these adapted animals were transferred to a solution that did not contain dopamine, they laid eggs at an abnormally high rate, suggesting that they had become dependent on exogenous dopamine for the control of egg laying (Table 1). Adapted animals regained sensitivity to dopamine over the course of approximately 4 hours (Fig. 1*b*). Long-term exposure to serotonin also led to adaptation. Serotonin (3 mg ml$^{-1}$, 7.7 mM) initially stimulated egg laying; however, animals exposed to serotonin overnight accumulated unlaid eggs, and were unable to lay eggs in response to a fresh dose of serotonin (Table 1, Fig. 2*d*).

TABLE 1 Adaptation to dopamine and serotonin

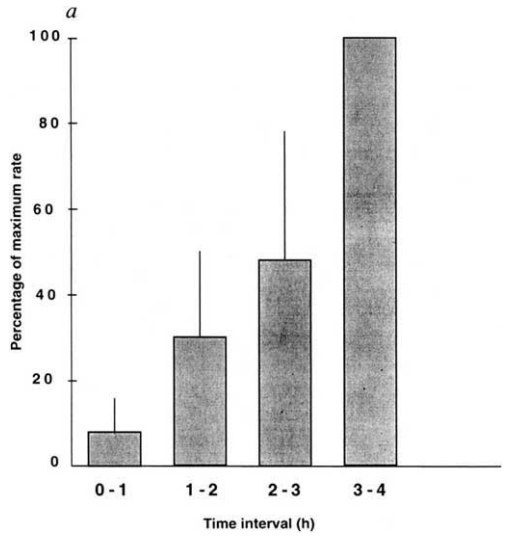
| Experiment | Strain genotype | Pretreatment | Test conditions | Eggs laid (eggs per worm per hour) | Percentage active |
|---|---|---|---|---|---|
| 1 | wild-type | no drug | no drug | 2.5 [±1.1] | 100 |
| | wild-type | no drug | 6 mg ml$^{-1}$ dopamine | 0.5 [±0.1] | 0 |
| | wild-type | 3 mg ml$^{-1}$ dopamine | 6 mg ml$^{-1}$ dopamine | 2.5 [±1.7] | 100 |
| 2 | *egl-1(n987)* | no drug | no drug | 0.6 [±0.2] | |
| | *egl-1(n987)* | no drug | 3 mg ml$^{-1}$ serotonin | 45.2 [±6.8] | |
| | *egl-1(n987)* | 3 mg ml$^{-1}$ serotonin | 3 mg ml$^{-1}$ serotonin | 0.8 [±0.8] | |
| 3 | wild-type | no drug | no drug | 3.6 [±2.8] | |
| | wild-type | 3 mg ml$^{-1}$ dopamine | no drug | 9.8 [±2.8] | |

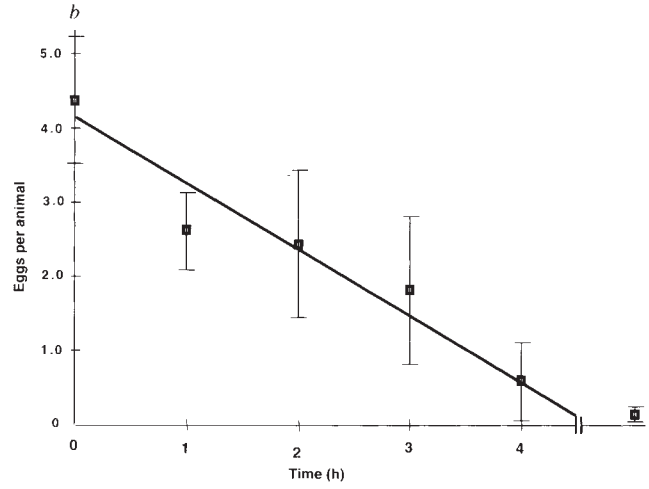
Effects of adaptation on egg laying and locomotion. Wild-type or *egl-1* adult hermaphrodites were grown overnight on 1.5% agar plates spread with *Escherichia coli* strain OP50, and with dopamine hydrochloride or serotonin creatinine sulphate added at the indicated concentrations (2 mM acetic acid was added to dopamine plates to stabilize the dopamine). To eliminate effects of endogenous serotonin, *egl-1* mutants, which lack HSN neurons, were used for the serotonin experiment. After overnight incubation in the presence or absence of drug, 10 animals were transferred to freshly poured test plates containing dopamine or serotonin as indicated, and were allowed to lay eggs at room temperature. Eggs were counted after 45 minutes for experiment 1, 1 hour for experiment 2, and 30 minutes for experiment 3. These data represent the mean rate of egg laying in 4 or more independent experiments (experiment 3 involved 11 independent trials). The sample standard deviation of these data is indicated in brackets.

We identified adaptation-defective mutants by looking for animals that remained sensitive to 3 mg ml$^{-1}$ dopamine after prolonged treatment. One of the mutants identified in such a screen, *mu74*, was found to be a recessive allele of the previously identified gene *unc-2* (refs 6, 7). The acute response of *unc-2(mu74)* animals to dopamine was essentially wild type (Fig. 2*a*), yet they were severely defective in desensitization; nearly all *unc-2(mu74)* animals treated with 3 mg ml$^{-1}$ dopamine failed to recover the ability to move after 14 hours (Fig. 2*b*). Thus the *unc-2(mu74)* mutation appeared to disrupt adaptation specifically. We tested the desensitization phenotypes of four other *unc-2* alleles: three alleles, *e55*, *e97* and *e129*, also had a strong dopamine adaptation-defective phenotype; a fourth allele, *e2379*, had only a subtle defect in dopamine adaptation (Fig. 2*b*). We found that *unc-2* mutants also exhibited several distinctive behavioural abnormalities, including constitutive egg laying (Fig. 2*c*) and slow, kinking movement. This behavioural phenotype mimicked the effect of serotonin treatment on wild-type animals, raising the possibility that *unc-2* mutants might also be unable to adapt to endogenous serotonin. The *unc-2* animals continued to lay eggs in response to serotonin even after overnight treatment (Fig. 2*d*); thus the *unc-2* gene appeared to be required for adaptation to serotonin as well as dopamine.

To understand the biochemical function of the *unc-2* gene product, we cloned the *unc-2* gene (Fig. 3). We found that *unc-2* encodes a predicted polypeptide product with high sequence similarity to the $\alpha$-1 subunit of voltage-sensitive calcium channels[8–11]. Although this protein did not appear to be the precise counterpart of a particular mammalian channel subtype, its sequence was more similar to neuronal $\alpha$-1 subunits of the A, B and E (non-L-type) classes than to channel subunits of the C, D, and $L_{skel}$ (L-type) classes. The *unc-2(mu74)* mutation deleted a predicted transmembrane $\alpha$-helix in the last of four membrane-spanning domains in the putative ion pore[12]; thus the mutant protein should have decreased channel function (Fig. 3). Interestingly, a mutation in the gene *unc-36*, which encodes a putative voltage-gated calcium-channel $\alpha$-2 subunit (L. Lobel and H. R. Horvitz, personal communication), produces essentially the same phenotype as mutations in *unc-2*; we have found that *unc-36* mutants are also defective in dopamine adaptation (W.R.S. and C.J.K., results unpublished). Thus, *unc-2* and *unc-36* may encode subunits of a voltage-gated calcium channel that participates in adaptation to dopamine and serotonin.

How might this channel act within the *C. elegans* nervous system to mediate behavioural plasticity? We focused initially on serotonin adaptation and egg-laying behaviour. Anatomical and pharmacological data implicate two classes of neurons, one of them serotonergic, in the control of egg laying. The two HSN motor neurons, which synapse onto the vulval muscle, are though to release serotonin to activate muscle contraction[13,14]. Six additional, ventral type C (VC), neurons receive input from HSN, and provide synaptic output to the vulval muscles[13]; both



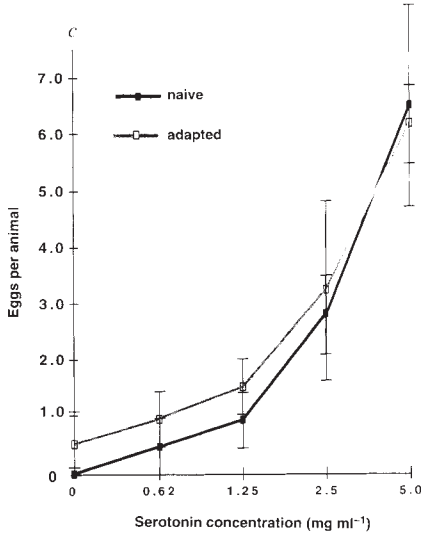




FIG. 1 Adaptation to dopamine and serotonin. *a*, Time course of dopamine adaptation. Animals were placed on 1.5% agar plates containing 3 mg ml$^{-1}$ dopamine at 20 °C; eggs were counted at 1-h intervals until the maximum rate of egg laying (mean = [4.0]) was reached. Data represent the mean percentage of the maximum rate for the indicated time interval. Mean and sample standard deviations were calculated from 4 populations of 10 animals each. *b*, Time course of dopamine resensitization. Animals were adapted overnight on 1.5% agar containing 3 mg ml$^{-1}$ dopamine as above. Adapted animals were then transferred to 1.5% agar containing 3 mg ml$^{-1}$ dopamine as above. Adapted animals were then transferred to 1.5% agar containing 3 mg ml$^{-1}$ dopamine as above. Adapted animals were then transferred to 1.5% agar plates lacking dopamine. At the indicated time points, groups of 10 animals were moved back to plates containing dopamine (4.5 mg ml$^{-1}$) and allowed to lay eggs. Each point and error bar indicates the mean and sample standard deviation, respectively, of 4 sets of 10 animals. *c*, Serotonin response of dopamine-adapted animals. To determine whether dopamine adaptation affected the response to serotonin, animals were grown overnight on 1.5% agar plates in the presence (adapted) or absence (control) of 3 mg ml$^{-1}$ dopamine as above. The ability of serotonin to stimulate egg laying was assayed in liquid culture as described[14]. Points and error bars indicate the mean and sample standard deviation of 3 sets of 10 animals. In all experiments, low-calcium agar was used, as calcium inhibits dopamine response in our assay.

the VCs and the HSNs produce a FMRFamide-like neuropeptide that can facilitate the ability of serotonin to stimulate egg laying[15]. *In situ* hybridization experiments indicated that *unc-2* message is expressed in both the HSN and VC neurons; in contrast, *unc-2* expression was not detected in the egg-laying muscles (Fig. 4*a*). Functional evidence that Unc-2 protein acts in neurons was obtained through analysis of mosaic animals[16] that were composed of both wild-type and *unc-2* mutant cells. In these mosaic animals, constitutive egg-laying behaviour correlated with lack of a functional *unc-2* gene in neuronal lineages, suggesting that the *unc-2* gene product is required in neurons to regulate egg laying (Fig. 4*b*).

In principle, the constitutive egg-laying behaviour of *unc-2* mutants could result from elevated release of serotonin from the HSNs, or from an abnormally sensitive response to serotonin by the egg-laying muscles. To investigate these possibilities, we eliminated the HSN neurons (the endogenous source of serotonin) using a mutation in the gene *egl-1* (ref. 14), which causes them to undergo cell death. If the constitutive egg-laying phenotype of *unc-2* mutants results exclusively from inappropriate serotonin release by the HSNs, then once the HSNs have been eliminated, *unc-2* and wild-type animals should exhibit the same response to serotonin. Alternatively, if the *unc-2* mutation causes an increased response to serotonin, the *unc-2* mutant lacking the HSNs should be serotonin hypersensitive. In fact, this was the case; the *egl-1*; *unc-2* double mutant failed to lay eggs in the absence of serotonin, but was sensitive to nearly tenfold lower concentrations of serotonin than either

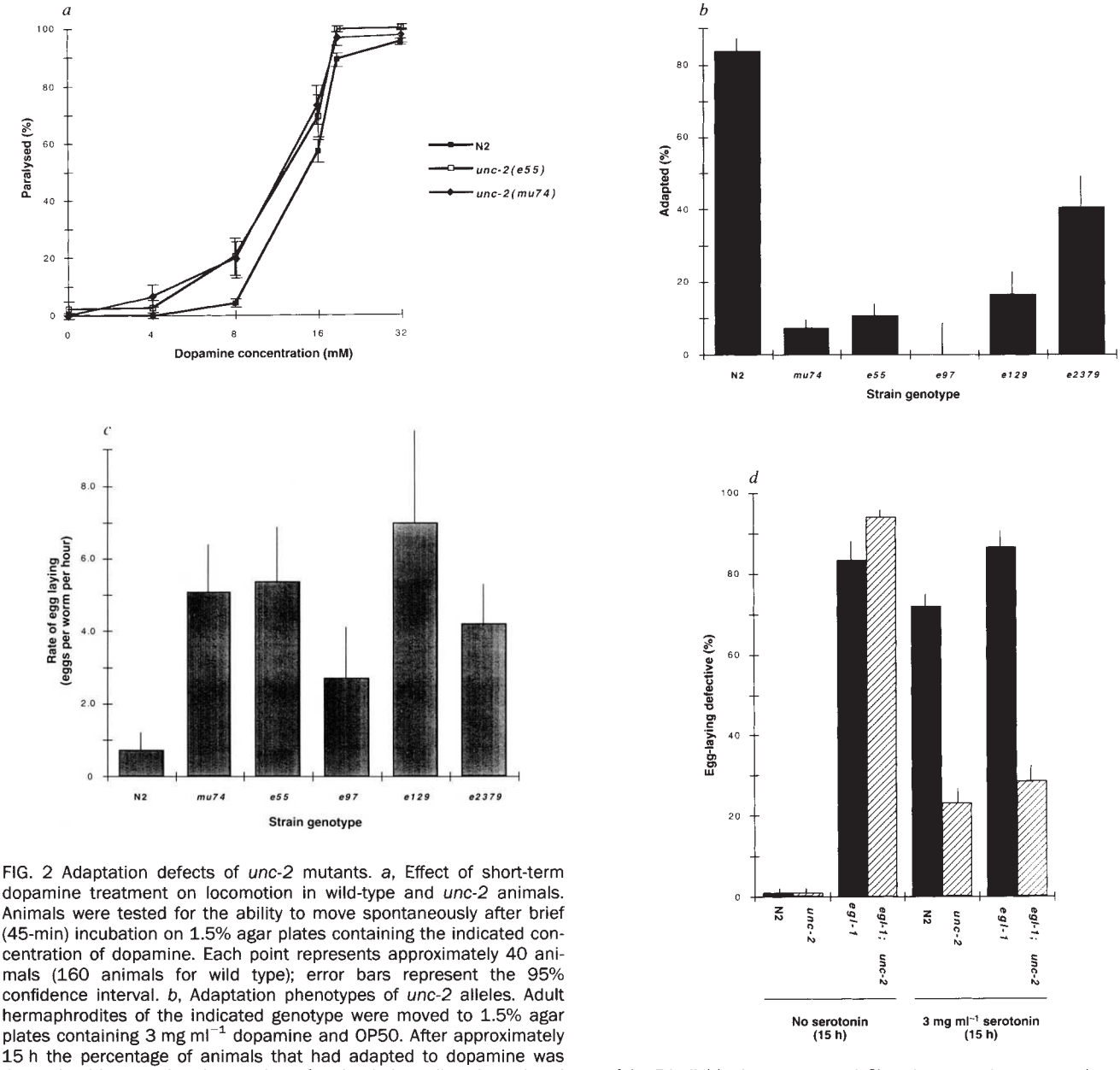



FIG. 2 Adaptation defects of *unc-2* mutants. *a*, Effect of short-term dopamine treatment on locomotion in wild-type and *unc-2* animals. Animals were tested for the ability to move spontaneously after brief (45-min) incubation on 1.5% agar plates containing the indicated concentration of dopamine. Each point represents approximately 40 animals (160 animals for wild type); error bars represent the 95% confidence interval. *b*, Adaptation phenotypes of *unc-2* alleles. Adult hermaphrodites of the indicated genotype were moved to 1.5% agar plates containing 3 mg ml$^{-1}$ dopamine and OP50. After approximately 15 h the percentage of animals that had adapted to dopamine was determined by counting the number of active (adapted) and paralysed (non-adapted) animals. Error bars represent the 95% confidence interval. The following numbers of animals were tested: N2, 105; *mu74*, 138; *e55*, 75; *e97*, 31; *e129*, 36; *e2379*, 32. *c*, Constitutive egg laying in *unc-2* mutants. Egg-laying rates for wild-type and mutant animals were determined during a 1-h incubation in liquid culture as described[14]. Bars represent the overall mean rate from 5 experiments of 10 animals each; error bars indicate the sample standard deviation of the 5 individual mean rates. *d*, Chronic serotonin response in *unc2*(+) and *unc-2*(e55) animals. Young adult hermaphrodites were incubated overnight on 3 mg ml$^{-1}$ serotonin as in Table 1. After approximately 15 h, animals that had accumulated unlaid eggs were scored as egg-laying defective. Each bar represents the percentage of egg-laying defective animals among at least 65 animals. Error bars indicate 95% confidence intervals for the difference between the *unc-2* mutant and *unc-2*(+) strain under each condition.

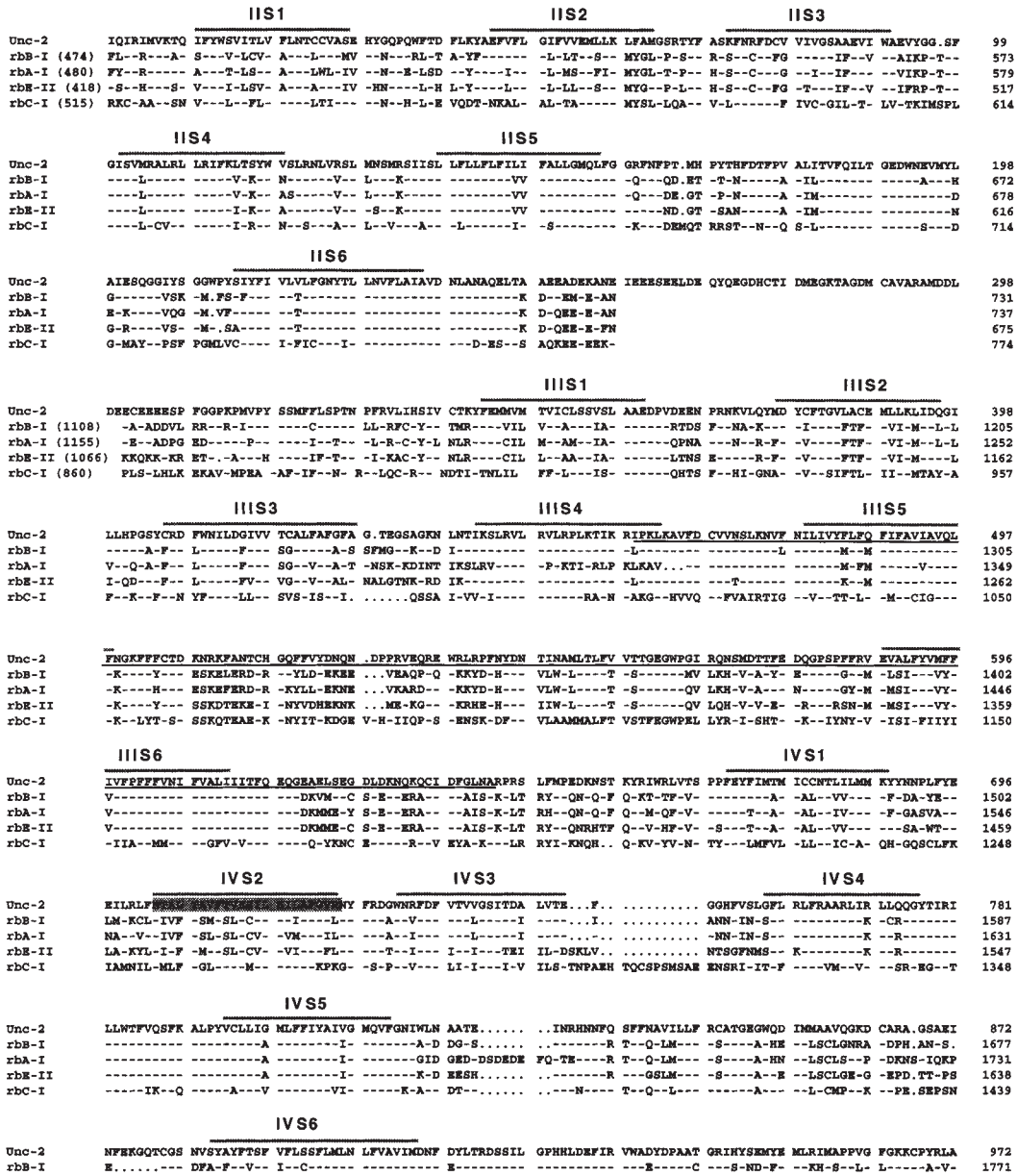
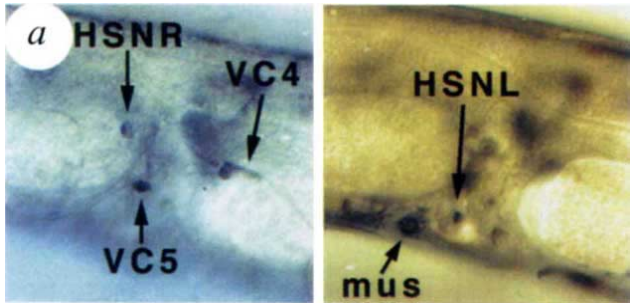
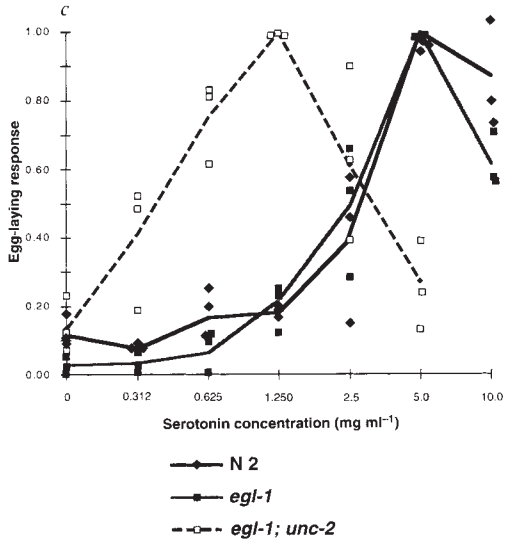
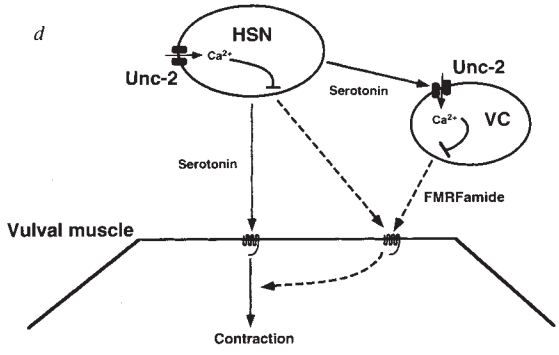
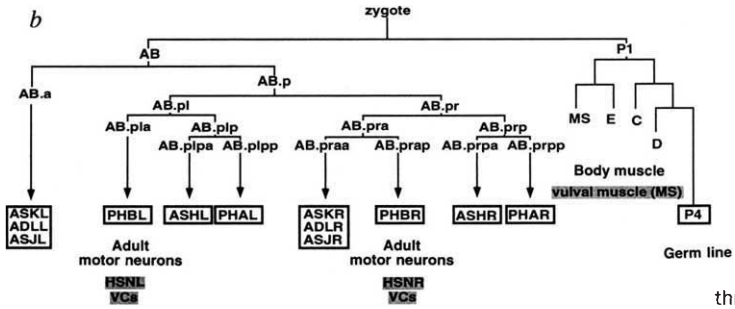



FIG. 3 Sequence similarity between Unc-2 and vertebrate calcium-channel proteins. Predicted sequence of Unc-2 is indicated on the top line; the corresponding regions of the rbB-I (rat brain B-I), rbA-I, rbE-II and rbC-I genes[8–11], encoding rat calcium-channel $\alpha$-1 subunits of the B, A, E and C classes, respectively, are displayed below (dashes indicate amino-acid identities). Overall amino-acid identities between Unc-2 and each rat channel protein are as follows: rbB-I, 640/1053 (61%); rbA-I, 636/1053 (60%); rbE-II, 627/1053 (60%); rbC-I, 478/1053 (45%). The transmembrane $\alpha$-helices comprising membrane domains II, III, and IV are indicated; sequences encoding the amino terminus of Unc-2 have not yet been identified. The region that is absent in *unc-2*(*mu74*) is shaded; the region encoded by the *in situ* probe B is underlined. Cloning of *unc-2* was done as follows: the *unc-2*(*mu74*) mutation was generated using the mutagen trimethylpsoralen, which often generates small deletions[17]; *unc-2* had previously been mapped to a narrow region of the *C. elegans* physical map[18,19]. We used a panel of cosmids containing DNA from this region to probe blots of *unc-2*(*mu74*) and wild-type DNA, and found that the cosmid T02C5 detected a *mu74*-specific deletion. This cosmid was injected into the germ line of *unc-2*(*e55*) hermaphrodites along with a cosmid containing the dominant cotransformation marker *rol-6*. Several of the *unc-2* phenotypes (kinking movement, constitutive egg laying, and long body length) were rescued in 3 of the 7 transformed animals expressing the *rol-6* phenotype, indicating that T02C5 contained *unc-2*. The *unc-2* DNA sequence was determined by sequencing both strands of a 7.3-kb region from the cosmid T02C5, which contained the *unc-2*(*mu74*) deletion. The sequence of the *unc-2*(*mu74*) mutant allele was determined by cloning polymerase chain reaction (PCR)-amplified DNA from mutant animals into the vector pCRII (Invitrogen), and sequencing the resulting plasmids. The GenBank accession number for the *unc-2* DNA sequence is U25119.




| Mosaic type | Duplication absent in | Inferred loss |
|---|---|---|
| Egl-C/Kinker | PHBL or PHAL: 4<br>PHBR or PHAR: 1<br>all stain: 2 | AB.pla or AB.plpp<br>AB.prap or AB.prpp<br>undetermined |
| Sluggish | P4-: 2<br>all stain: 2 | P1<br>undetermined |
| Wild-type | ASKL, ADLL, ASJL-: 1<br>ASHL, PHAL-: 2<br>PHAL or PHBL: 2<br>ASHL: 1<br>ASHR: 1 | AB.a<br>AB.plp<br>AB.pla or AB.plpp<br>AB.plpa<br>AB.prpa |

FIG. 4 Site of Unc-2 action in the control of egg laying. *a*, Pattern of *unc-2* mRNA expression in *C. elegans* hermaphrodites. Animals were fixed on polylysine slides, hybridized with a single-stranded digoxygenin (DIG)-labelled DNA probe, incubated with alkaline phosphatase-conjugated anti-DIG IgG (Boehringer), and stained with NBT/BCIP as described[20,21]. Anti-sense probes were generated as described from two regions of the *unc-2* gene (probe A was complementary to the region encoding amino acids 46–215, probe B to the region encoding amino acids 459–642); a control sense probe was also generated (encoding amino acids 1–169). Using probe B, *unc-2* message was detected in both the HSN and VC neurons. Expression was also observed in the body wall muscle (mus) and in a subset of the neurons of the ventral nerve cord and the head (not shown). Similar staining was observed with probe A (not shown); no staining was observed with the sense probe. In principle we cannot rule out the possibility that these probes could detect the messages of other, closely related, calcium-channel proteins; however, Southern blots using probe B sequences do not detect significant hybridization to genes other than *unc-2*. *b* Analysis of putative *unc-2* mosaics. We identified potential mosaics using an *unc-2* mutant strain carrying an intact copy of the *unc-2* gene on an unstable free duplication (genotype: *him-5* (*e1490*); *unc-2*(*e55*) *osm-5*(*p813*); *yDp16*). Mitotic loss of the duplication during development results in mosaic animals containing clones of genetically mutant cells in an otherwise wild-type organism. Potential mosaics were identified on the basis of partial *unc-2* mutant phenotypes; for example, one class of animal showed kinking movement and constitutive egg laying (Egl-C), but was not sluggish, whereas a second class was sluggish, but showed coordinated movement and normal egg laying behaviour. Because the *C. elegans* cell lineage (outlined above) is invariant, and known in detail[22], a cell-autonomous genetic marker can be used to infer the point of duplication loss, and thus to identify the cells that are genetically mutant. In this way we determined that most if not all of the Egl-C Kinkers had lost the duplication containing the functional *unc-2* gene in descendants of the cells AB.pl or AB.pr. Both these cells are precursors of neuronal cells, including the HSN, VC and adult motor neurons. In contrast, the Sluggish mosaics appeared to have lost the duplication in P1 or its descendents, which give rise to body wall and vulval muscle cells. The lower part indicates the cells in whose descendants the duplication was lost for each class of mosaics.

METHODS. We used the *osm-5* gene (like *unc-2*, *osm-5* is present on the duplication yDp16 (ref. 23)) to score for the presence of the duplication in the cells of the amphid (ASHL, ASHR, ASJL, ASJR, ASKL, ASKR, ADLL and ADLR) and phasmid (PHAL, PHBL, PHAR, PHBR) sensilla; we also assayed for the presence of the duplication in the cell P4, the germline precursor cell, by scoring for transmission of the duplication to the progeny of the mosaic animal. The *osm-5* phenotype was scored as described[16]; briefly, animals were stained with DiI on 1.5% agar plates, and staining of the amphid and phasmid cells was visualized by fluorescence. The patterns of staining seen in the duplication-bearing strain indicated that a wild-type copy of *osm-5* was required autonomously in the sensory cells for staining with DiI (ref. 16); thus the *osm-5* genotype of a particular sensory cell could be determined.

Egl-C Kinkers were identified as kinkers that laid early (16-cell or earlier) embryos; wild-type mosaics laid normal or late-stage embryos and had approximately wild-type movement. *c*, Acute response of wild-type (N2), *egl-1*(*n987*) and *egl-1*(*n987*); *unc-2*(*e55*) strains to serotonin. Egg-laying assays were performed in liquid culture as described[14]; eggs were counted after 30 min incubation. Because the strains accumulate different numbers of eggs in the absence of drug, the dose/response curves were normalized by dividing the total eggs laid at a particular dose by the number laid by that population at the optimal dose. The following represents the mean rate of egg laying at the maximal dose: N2, 4.4 eggs per animal per hour (at 5 mg ml$^{-1}$); *egl-1*(*n987*), 10.9 (at 5 mg ml$^{-1}$); *egl-1*(*n987*); *unc-2*(*e55*), 9.0 (at 1.25 mg ml$^{-1}$). Each point represents a single trial of 10 animals; the line traces the mean of these three trials. *d*, Model for the role of Unc-2 in the control of egg laying: *unc-2* is expressed, and may act, in the VC and HSN neurons. An *unc-2* mutation increases serotonin responsiveness, and blocks desensitization, even in the absence of HSN. Because both the VCs and HSNs express a FMRFamide-like peptide that can potentiate serotonin response, one hypothesis to explain the *unc-2* mutant phenotype is that Unc-2 is a subunit of a voltage-gated calcium channel that negatively regulates FMRFamide release. Serotonin adaptation may therefore involve activation of an Unc-2-dependent calcium influx in the VCs and/or HSNs, which inhibits the release of FMRFamide and thus decreases the responsiveness of the vulval muscles to serotonin.

the *egl-1* single mutant or the wild type (Fig. 4*c*). Moreover, whereas the *egl-1* single mutant still adapted to serotonin, the *egl-1*; *unc-2* double mutant was strongly adaptation defective (Fig. 2*d*). Thus *unc-2* mutants appear to lay eggs constitutively at least in part because their egg-laying muscles are hypersensitive and fail to adapt to endogenous serotonin. Where might the Unc-2 protein act to regulate egg laying? Mosaic analysis suggested that Unc-2 functions in neurons, yet the *egl-1* experiment demonstrated that Unc-2 does not require the HSNs to promote serotonin adaptation. An attractive hypothesis to explain these data is that Unc-2 modulates the release of FMRFamide, which can potentiate serotonin response from the VCs, and perhaps from the HSNs as well. Serotonin adaptation could occur if activation of the Unc-2 calcium channel causes an inhibition of FMRFamide release and thus a decrease in serotonin response (Fig. 4*d*).

In summary, we have shown that a voltage-gated calcium channel appears to be required for adaptation to dopamine and serotonin, and for determining the postsynaptic threshold for serotonin response. The *unc*-2 gene, which encodes the $\alpha$-1 subunit of this channel, is expressed in neurons that control egg-laying behaviour. We propose that, in these neurons, *unc*-2 may participate in serotonin adaptation by modulating the serotonin response, perhaps by controlling the release of a neuropeptide. The *unc-2* message is also expressed in several other neuronal and body muscle cells that may control response to dopamine; determination of the behavioural functions of *unc-2* in these cells may help to elucidate the mechanisms underlying dopamine adaptation. □

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1. Sulston, J., Dew, M. & Brenner, S. *J. comp. Neurol.* **163,** 215–226 (1975).
2. Horvitz, H. R., Chalfie, M., Trent, C., Sulston, J. & Evans, P. D. *Science* **216,** 1012–1014 (1982).
3. Avery, L. & Horvitz, H. R. *J. exp. Zool.* **253,** 263–270 (1990).
4. Loer, C. M. & Kenyon, C. J. *J. Neurosci.* **13,** 5407–5417 (1993).
5. Desai, C., Garriga, G., McIntire, S. & Horvitz, H. R. *Nature* **336,** 638–646 (1988).
6. Brenner, S. *Genetics* **77,** 71–94 (1974).
7. Avery, L. *Genetics* **133,** 897–917 (1993).
8. Snutch, T. P., Tomlinson, W. J., Leonard, J. P. & Gilbert, M. M. *Neuron* **7,** 45 (1991).
9. Starr, T. V. B., Prystay, W. & Snutch, T. P. *Proc. natn. acad. Sci. U.S.A.* **88,** 5621 (1991).
10. Dubel, S. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **89,** 5058–5062 (1992).
11. Soong, T. W. *et al. Science* **260,** 1133–1136 (1993).
12. Hofmann, F., Biel, M. & Flockerzi, V. *A. Rev. Neurosci.* **17,** 399–418 (1994).
13. White, J., Southgate, E., Thomson, N. & Brenner, S. *Phil. Trans. R. Soc.* B**314,** 1–340 (1986).
14. Trent, C., Tsung, N. & Horvitz, H. R. *Genetics* **104,** 619–647 (1983).
15. Schinkmann, K. & Li, C. *J. comp. Neurol.* **316,** 251–260 (1992).
16. Herman, R. K. *Genetics* **108,** 165–180 (1984).
17. Yandell, M. D., Edgar, L. G. & Wood, W. B. *Proc. natn. Acad. Sci. U.S.A.* **91,** 1381–1385 (1994).
18. Albertson, D. G. *Genetics* **134,** 211–219 (1993).
19. Zhao, C. & Emmons, S. W. *Nature* **373,** 74–78 (1995).
20. Seydoux, G. & Fire, A. *Development* **120,** 2823–2834 (1994).
21. Seydoux, G. & Fire, A. *Meth. Cell Biol.* (in the press).
22. Sulston, J. E., Schierenberg, E., White, J. G. & Thomson, J. N. *Devl Biol.* **100,** 64–119 (1983).
23. Akerib, C. C. & Meyer, B. *Genetics* **138,** 1105–1125 (1994).

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# Involvement of an ICE-like protease in Fas-mediated apoptosis

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**FAS is a type-I membrane protein that transduces an apoptotic signal[1,2]. Binding of Fas ligand or agonistic anti-Fas antibody to Fas kills the cells by apoptosis[3]. Studies in the nematode *Caenorhabditis elegans* have suggested that proteases such as interleukin-1β-converting enzyme (ICE) or the product of the *C. elegans* cell-death gene *ced*-3 are involved in apoptotic signal transduction[4]. The activity of ICE can be inhibited by the product of *crmA*, a cytokine-response modifier gene encoded by cowpox virus[5–7]. We report here that expression of *crmA* inhibits cytotoxicity induced by anti-Fas antibody or tumour necrosis factor (TNF). We have found a specific ICE inhibitor tetrapeptide (acetyl-Tyr-Val-Ala-Asp-chloromethylketone)[8,9] that also prevents apoptosis induced by anti-Fas antibody. These results suggest an involvement of an ICE-like protease in Fas-mediated apoptosis and TNF-induced cytotoxicity.**

Expression plasmids encoding *crmA*[10] and human *fas*[1] were introduced into Rat-2 (rat fibroblast) cells together with the neomycin-resistance gene. Among G418-resistant transformants, the Fas-expressing transformants were selected by flow cytometry analysis using mouse anti-human Fas antibody. Three clones (33, 45 and 48) were found to express human Fas on the cell surface (data not shown). Immunoprecipitation using anti-human Fas antibody confirmed the expression of Fas in these transformants (Fig. 1*a*). As found previously[1], immunoprecipitation gave two bands ($M_r$ 43K and 50K) for human Fas, which may reflect different degrees of glycosylation. The expression of *crmA* was then analysed by RNase protection assay using a $^{32}$P-labelled RNA fragment carrying the *crmA* gene (nucleotides 1,174 to 1,468). The control *crmA* RNA produced by *in vitro* transcription gave protected bands of 280, 250 and 150 bases (Fig. 1*b*). The upper band was of the size expected, but the other two bands may have been products of digestion at AU-rich regions of the *crmA* RNA. The same protected bands were observed with messenger RNAs from clones 33 and 48, but no such bands were detected with mRNA from the parental Rat-2 cells and clone 45.

The parental Rat-2 cell line and its transformants were then treated with the agonistic anti-human Fas antibody. As shown in Fig. 2*a*, the anti-Fas antibody had no effect on Rat-2 cells. However, clone 45, which expressed human Fas but not CrmA, was killed within 14 hours by the anti-Fas antibody in a dose-dependent manner in the presence of 50 ng ml$^{-1}$ actinomycin D. Almost all cells died within 10 hours of treatment with 1 μg ml$^{-1}$ of the anti-Fas antibody (Fig. 2*b*). However, the transformant clones 33 and 48 that expressed CrmA were resistant to the cytotoxic activity of the anti-human Fas antibody, and even survived incubation for 10 hours with 1 μg ml$^{-1}$ of anti-Fas antibody. The inhibition of Fas-mediated apoptosis by CrmA was dose dependent, that is, the transformed clones expressing small amounts of *crmA* mRNA were weakly protected against Fas-mediated apoptosis (data not shown).

TNF has cytotoxic activity in various cell lines. To examine whether an ICE-like protease is also involved in TNF-induced cytotoxicity, Rat-2 cells and its transformants were treated with TNF. The Rat-2 cells were killed by 250 ng ml$^{-1}$ of mouse TNF in the presence of 12.5 ng ml$^{-1}$ actinomycin D (Fig. 2*c*), although this process was much slower than Fas-mediated cytotoxicity (all cells were killed in ~60 hours). Clone 45, which expresses Fas but not CrmA, was killed by TNF treatment as efficiently as were the parental Rat-2 cells, but clones 33 and 48,

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